United States Environmental Protection Agency

LABORATORY ANIMAL PROJECT REVIEW

Please note:

1. All information in this LAPR is considered privileged and confidential by the IACUC and regulatory authorities.
2. Approved LAPRs are subject to release to the public under the Freedom of Information Act (FOIA). Do not include proprietary or classified information in the LAPR.
3. An approved LAPR is valid for three years.

LAPR Information

LAPR Title:	A Neuroendocrine Adverse Outcome Pathway Linking Environmental Stressors to Glucose Homeostasis in a Rat Model
LAPR Number:	20-04-001
Principal Investigator	Exemption 6
Author of this Document:	Exemption 6 /RTP/USEPA/US
Date Originated:	03/27/2017
LAPR Expiration Date:	04/30/2020
Agenda Date:	04/05/2017
Date Approved:	04/12/2017
Date Closed:	

APPROVALS

APPROVER	NAME	APPROVAL DATE	COMMENTS	
	Exemption 6 Exemption 6 Exemption 6 Exemption 6 TP/USEPA/US by Exemption 6 RTP/USEPA/US	04/12/2017	DMR	
	Exemption 6 Exemption 6 Exemption 6 RTP/USEPA/US by Exemption 6 /RTP/USEPA/US	04/12/2017	DMR	

Administrative Information

1. Project Title (no abbreviations, include species):
A Neuroendocrine Adverse Outcome Pathway Linking Environmental Stressors to Glucose Homeostasis in a Rat Model

Is this a continuing study with a previously approved LAPR?
No

2. Programatic Information

a. What Program does this LAPR support? Please provide the Research Program, Project, Task Number and Title.
ACE: 119, 158 and 242 (New projects: ACE PEP1 and PEP2)

b. What is the Quality Assurance Project Plan (QAPP) covering this project?
QAPP-NHEERL-RTP/EPHD/CIB/Exemption 6 2017-001-r0

3. EPA Principal Investigator/Responsible Employee:

Principal Investigator	*Phone Number*	*Division*	*Mail Drop*
Exemption 6	Exemption 6	EPHD	MD
	Lotus Notes Address	*Branch*	
	Exemption 6 /RTP/USEPA/US	CIB	

4. Alternate Contact:

Alternate Contact	*Phone Number*	*Division*	*Mail Drop*
Exemption 6	Exemption 6	EPHD	MD
	Lotus Notes Address	*Branch*	
	Exemption 6 TP/USEPA/US; Exemption 6 /RTP/USEPA/US	CIB	

SECTION A - Description of Project

1. Explain the study objective(s) in <u>non-technical language</u> such that it is understandable by non-scientific persons. <u>Explain how the benefits from the knowledge gained from this research outweigh the costs to the animals used in this research.</u> If this is a continuing study from a previous LAPR, briefly justify the continuation. Please spell out all acronyms and abbreviations with their initial use.

Previous studies from our lab and others have demonstrated that exposure to air pollutants such as ozone lead to metabolic alterations in humans in rats. For instance, we've found that acute ozone exposures in rats lead to disruptions in glucose homeostasis including hyperglycemia (high blood glucose levels) and glucose intolerance (the inability of the body to uptake glucose from the bloodstream). These changes coincide with ozone-induced increases in stress hormones (adrenaline and corticosterone) that impair glucose homeostasis by acting on metabolic tissues to mobilize glucose and fatty acids into the bloodstream via gluconeogenesis in the liver and fat breakdown in adipose tissue.

Our goal of this project is to use implantable glucose telemeters in rats to measure dynamic changes in blood glucose levels prior to, during, and following exposure to ozone. These real-time data will allow us to better understand the timing of when glucose alterations occur in the rat, which will help us further elucidate the mechanisms behind ozone-induced disruption in glucose homeostasis. Furthermore, these data will be used by computational modelers to help develop a quantitative adverse outcome pathway (qAOP) that will be used to explain how activation of stress response pathways by environmental exposures leads to metabolic alterations.

2. Scientific rationale for proposed animal use.

a. Why is the use of animals necessary?

The use of animals is necessary in order to understand the complex systemic changes in multiple organs that occur after exposure to pollutants in animal models. We have previously demonstrated that exposure of healthy rats to pollutants can produce profound systemic responses through the neuroendocrine stress pathway. An in vitro experimental approach would not be appropriate for determination of systemic changes in blood glucose levels during and after exposure to inhaled ozone nor would it be appropriate for assessing neuroendocrine stress effects on multiple organs. Following a bibliographic search in Pubmed, no validated accepted non-animal methods have been identified to properly mimic inhalation exposures and in-life assessment of changes in circulating glucose. Further, the use of an animal model is essential for the new glucose telemetry technologies for glucose measurement.

b. Justify the species requested:

National Institute of Health guidelines recommend the use of rats to study human cardiovascular and metabolic diseases. The rat has also been a preferred animal model for the study of cardiovascular injury from air pollution. Moreover, systemic disorders are better modeled in rats than in mice or in lower vertebrates. Due to the longstanding use of rats for toxicological, cardiovascular, and metabolic studies the necessary databases, reagents, and species-specific assays have been developed, verified to be accurate, and are commercially available. Historical toxicology data are available for rats to correlate findings of air pollution health effects. We have done a number of toxicological studies using Wistar Kyoto (WKY) rats. Since the studies anticipated under this LAPR involve continuation of our previous studies using WKY rats for examining metabolic health effects, we propose using this rat strain to understand how stress response mediates systemic and pulmonary effects of ozone.

3. How was it determined that this study is not unnecessary duplication?

Pubmed and literature searches in Google Scholar performed in March 2017 failed to identify any published studies that investigated in-life changes in blood glucose after ozone exposure using radiotelemetry implantation of glucose monitors. The search terms included "ozone", "glucose monitors", and "rat" or "mouse." An additional search that included "air pollution" and "glucose monitors" also failed to identify any published papers. The glucose in-life monitoring is a new technology and few studies in the field of medicine have used these devices. No air pollution studies to our knowledge have used this technology.

<u>SECTION B - In Vivo Procedures</u>

1. Briefly describe the experimental design. Include descriptions of the age, weight and sex of the animals. Supplementary information may be attached at the end of the LAPR, but please include

critical information within the body of the LAPR.

This project will include two experiments: 1) The first one will involve surgical implantation of glucose monitors for telemetry, and subsequent exposure of rats to ozone to determine the temporality of circulating glucose changes during and post ozone exposure. 2) The second experiment will use a separate cohort of naïve rats exposed to air or ozone and will involve the measurement of circulating stress hormones and metabolic biomarkers as well as tissue collection for determining gene and protein changes at times when circulating glucose changes begin to increase, peak, and return back to baseline levels.

Experiment #1:

Twenty male WKY rats (12-14 weeks old, 300-350g) will be used. Baseline whole-body plethysmography will be performed to exclude rats exhibiting high PenH levels (an indication of labored breathing and spontaneous cardiac hypertrophy, generally observed in 5-20% of WKY rats). Additional rats will be made available to the surgeon for any potential surgery related complications, as generally recommended for experiments involving animal surgery. Therefore, 20 rats will be ordered for 8 successful surgical implantations and to ensure that we have enough remaining non-telemetered cage control rats to double house with our telemetered rats. Surgery of all animals (n=8) will take place on Day 1 of Week 1. Following a 6 day recovery period, 4h exposures to 0.0, 0.2, 0.4, or 0.8 ppm ozone once per week will take place over a 4 week period in the mornings on Day 1 of each week using a crossover exposure design to ensure that all 8 implanted animals are exposed to each concentration once. During the 4 weeks of exposure, we will continuously monitor and collect data for blood glucose levels, core body temperature, and activity level prior to, during, and following ozone exposure.

The glucose monitors are guaranteed to provide data for at least 4 weeks but, generally the reagents can last for up to 6-8 weeks as per the experience of the representative. To make use of all time available for glucose monitoring, we will use the same implanted animals and expose them to either 0.0 or 0.8 ppm ozone (n=4/concentration) for a single 4h exposure on Day 1 of Week 6 and 7. On Week 6, we will conduct a glucose tolerance test (GTT) immediately following the acute exposure. On Week 7, we will conduct a pyruvate tolerance test (PTT) immediately following the acute exposure. If the glucose monitors are still viable, we will use the same implanted animals and expose them to either 0.0 or 0.8 ppm ozone (n=4/concentration) on Week 8 for a series of 4h exposures for 4 consecutive days on Day 1 - 4. Immediately following the 1st ozone exposure on Week 8, we will conduct an insulin tolerance test (ITT). Animals will be necropsied on Day 5 on Week 8 for blood sample and tissue collection.

Experiment #2;

The purpose of this study is to elucidate mechanisms behind ozone-induced metabolic alterations by constructing a time line of events for stress hormone release leading to tissue metabolic perturbations using naïve male WKY rats (12-14 weeks old, 300-350g).

Immediately following ozone exposures of different durations, animals will undergo euthanasia and necropsy to collect blood samples, metabolic tissues, and brains in an attempt to elucidate the temporal mechanisms leading to the disruption in glucose homeostasis. Rats will be exposed for 30 min, 1h, 2h, or 4h to air or 0.8 ppm ozone. Immediately post exposure, blood samples will be collected and tissues (lung, brain, liver, adipose, muscle, thymus, spleen, and bone marrow) snap frozen and stored for future assessment. These data will be matched with live glucose collection to assess the temporal relationship to circulating glucose, stress hormones and peripheral tissue changes. This information can be incorporated in quantitative AOP. This temporal assessment will require 64 rats (n=8 rats/group x 2 exposures (air or ozone) x 4 time points).

2. Justify the number of animals. Include explanation (e.g., biological, statistical, regulatory rationale) for the number of animals needed for each treatment group, and the overall number requested for the duration of the LAPR.

We request 20 rats for first surgery experiment to have 8 successful implants and to ensure that our implanted rats are double housed with a non-telemetered cagemate to avoid any potential stress occurring as a result of being single housed. Our past years of studies using male WKY rats have shown that 5-20% of rats exhibit spontaneous cardiomyopathy, often having secondary pulmonary complications. Since there is a

great correlation between enlarged heart and high baseline PenH value (an indicator of labored breathing) obtained using whole body plethysmography prior to the start of experiment, we anticipate excluding a maximum of 2-4 of these rats from these 20. Thus, the remaining rats will be sufficient for obtaining 8 successful surgeries. The non-implanted rats will also be used for collecting baseline tissue and serum samples from this experiment. The 8 rats undergoing surgeries will all be exposed to ozone and will be considered under category E. Other 12 will be in category C.

For the second experiment, we anticipate using 6 rats/group with 2 extra rats/group being requested to obtain a sufficient number of rats such that those with high PenH levels at baseline can be excluded from the study a priori. The remaining extra rats with normal PenH will be used as cage controls. The numbers are selected in order to obtain the anticipated effect size to assess serum chemistry and tissue changes, and corroborate with the experimental design in our prior ozone studies for appropriate comparison of results. Of 64 rats (non surgery group), 24 will be exposed to ozone and are considered category E. The other 40 will be considered category C.

3. State how many animals over the study period are expected to be used under the following three categories of pain/distress (USDA nomenclature as defined in the instructions): Please enter numbers only.

Categories	*Adults*	*Offspring*
C) Minimal, transient, or no pain/distress:	52	
D) Potential pain/distress relieved by appropriate measures:		
E) Unrelieved pain/distress:	32	

4. Does this LAPR include any of the following:

☐ Restraint (>15 Minutes) ☒ Survival surgery

☒ Food and/or water restriction (>6 Hours) ☐ Non-survival surgery

a. Please provide a scientific justification. Describe how animals will be monitored, how health status will be tracked, and what records will be maintained.

Our goal of this project is to use implantable glucose telemeters in rats, which requires survival surgery, to measure dynamic changes in blood glucose levels prior to, during, and following exposure to ozone. These real-time data will allow us to better understand the timing of when glucose alterations occur in the rat, which will help us further elucidate the mechanisms behind ozone-induced disruption in glucose homeostasis. Furthermore, these data will be used by computational modelers to help develop a quantitative adverse outcome pathway (qAOP) that will be used to explain how activation of stress response pathways by environmental exposures leads to metabolic alterations.

Static glucose measurements post-exposure have been conducted in several studies using blood collected from a single tail prick. Although this is an acceptable, safe way to measure blood glucose levels post-exposure, it does not provide the continuous, dynamic blood glucose levels prior to, during, and following pollution exposure. Implanting these glucose monitors is the only means available for these types of measurements. Therefore, it is necessary to use these HD-XG glucose telemeters for understanding the mechanisms by which pollutants alter glucose homeostasis.

Food and water restriction is necessary (~6h) in Experiment #1 to obtain stabilized baseline values for metabolites, which are highly influenced by food intake and in Experiment #2 to ensure animals across the different time course exposures (i.e. 30 min, 1h, 2h, and 4h) are at a consistent fasting state for comparisons of measurements in metabolic tissues and markers.

The glucose monitors will be surgically implanted in 8 rats. After surgery, rats will be continuously monitored until fully awake and placed on a heating pad and/or other heat source combination such as forced air warmer, space gels, bubble wrap, and infrared heating pad to maintain body temperature. Powdered food provided in feeding cups and regular feed offered in bowls on the floor and the food hopper will be provided post-surgery for all animals for at least 7-10 days post-surgery. Although no deaths are anticipated from this procedure, rats will be closely monitored during the postoperative period. After the surgery, animals will have rest for 6 full days before the beginning of exposure. No other special husbandry requirements are needed for implanted rats. Animals will be provided regular tap water in water bottles or automatic water supply. We will closely monitor these rats and

will provide additional analgesic treatment as recommended by attending veterinarian.

Exemption 6Exemption 6Exemption 6Exemption 6 will be monitoring animals twice daily after their surgery (during recovery and experimentation periods including weekends). It is not expected that animals will suffer dehydration or weight loss issues based on the type of implantation. The attending veterinarian will be notified immediately if any surgery-related complications are noted during experimentation.

Data are recorded both on PC and in loose leaf papers on a preset spreadsheet in the lab during the necropsy and cell counting. These files are maintained in Exemption 6 in Exemption 6 office. Some study protocols, data copies obtained from Exemption 6, and other individuals are maintained in Exemption 6. Exemption 6 will lead the study and keep all records regarding the surgery, ozone exposures, telemetry data collection, and molecular assessment on tissues in the laboratory notebook in her office room Exemption 6. Records will be kept in accordance with document guidelines in Exemption 6 and ORD's Policy for Paper Laboratory Records.

Folders with original data sheets and relevant study related information are maintained in B564. Additional folders with some original data are stored in Exemption 6 office Exemption 6.

5. Category C procedures. Describe each procedure separately, include details on the following:

a. Treatments (e.g., dosages, duration of exposure, route, volume, frequency):

Glucose injection for glucose tolerance testing (GTT): During GTT, after the baseline blood glucose measurement is taken, pharmaceutical grade glucose will be injected intraperitoneally (maximum of 2 g/kg body weight/10 mL pharmaceutical grade saline). The 10 mL/kg body weight volume has been used in published studies for rats. We will purchase pharmaceutical grade sterile glucose solution and dilute to 20% using pharmaceutical grade saline. Glucose solution will be made fresh each time using new pharmaceutical grade saline vials. Sterile syringe and needles will be used for each rat for intraperitoneal injection. In our prior studies, animals showed no signs of peritonitis or infections in the abdomen after repeated GTT over a three month period. Their weight gains were not affected by GTT. The scientific staff involved in the study will watch for signs of abdominal pain such as lateral or vertical stretching, and weight loss daily until the necropsy. GTT will be performed once in 8 rats (Experiment #1, Week 6).

Pyruvate injection for pyruvate tolerance testing (PTT): During PTT, after the baseline blood glucose measurement is taken, sodium pyruvate in pharmaceutical grade saline will be injected intraperitoneally (1 g/kg body weight/2 mL pharmaceutical grade saline). The 2 mL/kg body weight volume has been used in published studies for rats. We will purchase pharmaceutical grade sterile solution to prepare 1 g/2 mL sodium pyruvate. Pyruvate solution will be made fresh using new pharmaceutical grade saline vials prior to its use. Sterile syringe and needles will be used for each rat for intraperitoneal injection. In our prior studies, animals showed no signs of discomfort after pyruvate injection at this concentration. The scientific staff involved in the study will watch for signs of abdominal pain such as lateral or vertical stretching. PTT will be performed once in 8 rats (Experiment #1, Week 7).

Insulin injection for insulin tolerance testing (ITT): During ITT, after the baseline blood glucose measurement is taken, HumulinR insulin (which comes as a solution of 100 U/mL) will be diluted in pharmaceutical grade saline and injected intraperitoneally (0.75 U/kg body weight/mL pharmaceutical grade saline). The 1 mL/kg body weight volume has been used in published studies for rats. We will purchase pharmaceutical grade sterile solution to prepare 1 U/ mL insulin. Insulin solution will be made fresh using new pharmaceutical grade saline vials prior to its use. Sterile syringe and needles will be used for each rat for intraperitoneal injection. In our prior studies, animals showed no signs of discomfort after insulin injection at this concentration. The scientific staff involved in the study will watch for signs of abdominal pain such as lateral or vertical stretching. We will use 0.75 U/kg insulin dose and only 6-7 hours fasting to avoid significant hypoglycemia during testing. Insulin injection is not likely to drop glucose level below 50 mg/dL based on the literature review; however, in case blood glucose levels approach 40 mg/dL or below, a bolus injection of pharmaceutical grade glucose solution (used in our GTT protocol) at 2 g/kg/10 mL is given to rats to prevent severe hypoglycemia. This protocol is also used in many studies involving examination of insulin resistance and no deleterious effects are expected. ITT will be performed once in 8 rats (Experiment #1, following the first ozone exposure in Week 8).

For all injections during GTT, ITT, and PTT, we will inject using 26-gauge x 3/8 inch needles. (Insulin will be

injected with 0.5 inch length needles).

b. Survival Blood Collections (method, volume, frequency):

For all rats undergoing GTT, 5 total blood samples at 1 microliter/sample will be taken for measuring glucose levels (n=8 in Experiment #1). For all rats undergoing PTT and ITT, 6 total blood samples at 1 microliter/sample will be taken for measuring glucose levels (n=8 in Experiment #1, once for PTT and once for ITT). Blood will be collected by pricking the tip of the tail with a 25-gauge sterile needle following wiping with an alcohol swab and clean dry gauze. About 1 microliter blood droplet will be brought into contact with the glucometer strip (attached to Bayer Contour Glucometer; 0.6 microliter of blood is aspirated in the strip). Glucose is measured within three seconds and recorded. Once baseline glucose is measured, pharmaceutical grade glucose (2 g/kg body weight/10 mL), pyruvate (1 g/kg body weight/2 mL), or insulin (0.75 U/kg body weight/mL) will then be injected intraperitoneally and blood glucose will be measured at 30 min intervals for 4-5 times in each rat.

-Blood glucose measured by tail prick at 0 min
-Glucose injected intraperitoneally right after the zero minute blood glucose testing
-Glucose measured by tail prick at 15 min (PTT and ITT only)
-Glucose measured by tail prick at 30 min
-Glucose measured by tail prick at 60 min
-Glucose measured by tail prick at 90 min
-Glucose measured by tail prick at 120 min

c. Testing methods (including non-stressful dietary restrictions/modifications, mild non-damaging electric shock):

Surgically implanted animals in Experiment 1 will be fasted during ozone exposure. The performance of GTT, PTT, and ITT after 4h of ozone exposure without food will constitute sufficient fasting time in implanted animals. Naïve animals in Experiment 2 will be fasted for 3-4 hours prior to start of ozone exposure and necropsies performed immediately post exposure.

Whole-body plethysmography using EMKA/Buxco system: Respiratory monitoring using whole body plethysmography measurements will be performed at baseline for all animals in both experiments (prior to surgery and/or ozone exposure in naïve rats). Breathing parameters are monitored in freely moving rats. Rats are placed in plethysmography chambers while pressure parameters are collected to compute breathing frequency, minute volume, respiratory time and enhanced pause (PenH). The rats are placed in a whole-body plethysmography for 5-10 min. We have routinely used this duration for acquisition of breathing parameters which has been adequate for stabilization and recording. No restraint or other stresses are involved in this process. This measurement allows in depth evaluation of lung health in unrestrained freely moving rats.

d. Animal restraint and confinement beyond routine housing and handling. Include a description of the type of restraint device, acclimation to device, duration of restraint:

None

e. Breeding for experimental purposes (e.g. length of pairing, number of generations):

None

f. Describe how animals will be identified and monitored. Include description of identification procedures. (For example, if transponders are used, how are the animals prepared?) Include frequency of observations and by whom:

Each individual animal is identified using a unique number in a given experiment. Each animal will be identified by a unique identification number marked using permanent marker on their tail and all cages will be labeled with details of animal numbers, treatments, and exposure conditions. The animals with telemetry implants will be continuously monitored during the day of surgery. During recovery period, animals will be monitored two times a day for any potential surgery-related complications. During exposure, **Exemption 6Exemption 6 Exemption 6** will monitor animals, at least once per hour for entire exposure duration. During post exposure periods, rats will be monitored by **Exemption 6Exemption 6Exemption 6** in the evening and then in the morning for visible signs of discomfort and weight loss. All animals will be monitored for signs of illness (huddling, isolation with ruffled coat, shivering, development of hindered movement, etc) and if any adverse effect is observed, we will consult with the staff veterinarian and follow the recommended protocol. Visual inspection of labored breathing and isolation will be carefully monitored. No significant weight loss due to ozone is expected in any of the experimental conditions.

6. Non-surgical Category D or E procedures. Describe each procedure separately, include details on the following (Also fill in Section B.9).

a. Treatments (e.g. dosages, duration of exposure, route, volume, frequency):

Ozone exposure at 0.8 ppm in WKY rats is classified as a Category E procedure. Rats will be exposed to 0.2, 0.4, or 0.8 ppm ozone, up to 4h/day, for either 1 or 4 consecutive days. In Experiment #1, a crossover design will be used, and so all rats will eventually be exposed to all concentrations of ozone including 0.8 ppm. Ozone at the 0.8 ppm concentration in WKY rats produce lung inflammation, hypothermia, and a stress response which resolves on its own after one day upon discontinuation of exposure.

During air or ozone inhalation, exposures will be for a maximum of 4h/day (whole-body). Rats are placed in individual stainless steel wire mesh cages (length, 27.3 cms; width, 14.6 cms, and height 17.75 cms), and food and water are withheld while the rats are being exposed. Ozone exposures will be done using whole body exposure system in large Hazelton 2000 Inhalation chambers (2 cubic meters internal volume, CH Technologies, Westwood, NJ) where each rat is placed in wire-mesh cages. The rats are weighed daily following each exposure and examined for any visible clinical signs of discomfort or poor health. The rats are also checked after each exposure when they are returned to home cages. All findings are recorded.

b. Blood Collection (Provide a description of the procedure including method, volume, and frequency if appropriate. Indicate if the procedure is survival or terminal. Include preparatory methods, descriptions of incisions, etc.):

c. Testing methods:

d. Restrictions placed on the animals' basic needs (e.g., food and/or water restriction, light cycles, temperature). Provide details regarding the length of restriction. Describe the method(s) for assessing the health and well-being of the animals during restriction. (Amount of food or fluid earned during testing and amount freely given must be recorded and assessed to assure proper nutrition.):

Food restriction for ~6h during ozone exposure and GTT, PTT, and ITT: The performance of these tests and the assessment of metabolic markers requires prior ~6h fasting in rodent studies to obtain stabilized baseline values for metabolites, which are highly influenced by food intake. GTT will be conducted in Week 6, PTT during Week 7, and ITT during Week 8 of glucose monitoring in Experiment #1. Animals will be fasted during 4h ozone exposure and then during ~2.5h of GTT, PTT, or ITT testing. Animals will be monitored hourly during ozone exposure for signs of discomfort and then continuously through testing.

In Experiment #2, naive animals will be fasted for 3-4 hours prior to start of ozone exposure and necropsies performed immediately post exposure. This is to ensure animals across the different time course exposures (i.e. 30 min, 1h, 2h, and 4h) are at a consistent fasting state for comparisons of measurements in metabolic tissues and markers.

e. Describe how animals will be monitored (e.g., frequency of observations, by whom):

The animals with telemetry implants that have undergone ozone exposure will be continuously monitored. During exposure, **Exemption 6Exemption 6Exemption 6Exemption 6** will monitor animals at least once per hour for entire exposure duration. During post exposure period of up to 20h, rats will be monitored by **Exemption 6Exemption 6Exemption 6** in the evening and then in the morning for visible signs of discomfort and weight loss. All animals will be monitored for signs of illness (huddling, isolation with ruffled coat, shivering, development of hindered movement, etc) and if any adverse effect is observed, we will consult with the staff veterinarian and follow the recommended protocol. Visual inspection of labored breathing and isolation will be carefully monitored. No significant weight loss due to ozone is expected in any of the experimental conditions.

f. Analgesia (Category D Procedures) - list drugs, dosages, route of administration and frequency:

No analgesia will be used for the procedures described in this section; however, analgesics will be used for surgical procedures as described in section 7.

g. If treatment-related deaths are expected, this must be thoroughly justified. Death as an endpoint is highly discouraged:

No procedure-related deaths are expected for this category.

7. Surgical Category D and E procedures. Indicate if the surgery is survival or terminal. Describe each surgical procedure separately, include details on the following (Also fill in Section B.9)

a. Complete description of surgical procedure including presurgical preparation, aseptic technique, surgical closure, etc:

Rats will be purchased and housed in EPA animal facility until they reach 12-14 weeks age. The surgeries will be performed at 12-14 weeks of age on animals that weigh between 300-350 g. The surgeries will be performed in the 3rd floor procedure rooms Exemption 6 Exemption 6. The room will be reserved in advance and the door signs will restrict entry of non-essential personnel to the suite during surgeries.

HD-XG Glucose Telemetry Implant: The HD-XG continuous glucose telemetry implant from Data Sciences International (DSI) is a device used to detect blood glucose, body temperature, and activity level measurements and transmit the data from within the animals via radio-frequency signals. Data is transmitted continuously and in real-time via a radio telemetry signal to a local receiver and collected in the Dataquest A.R.T. data acquisition system. The HD-XG glucose sensor is guaranteed to last for 28 days post-implantation but frequently functions for 6-8 weeks, with a battery life that frequently functions 8 weeks or longer. The sensor has a glucose range of 10-750 mg/dL and collects 50 blood glucose measurements/second. The device consists of an implant body that houses the reusable electronics module that translates the glucose fluctuations into digitized signals and transmits them to a receiver, a magnetically activated switch that allows the implant to be switched on or off, a battery that provides the power supply for the electronics module, and a suture rib that allows the surgeon to suture the implant securely in place at the implantation site. The device also consists of a glucose sensor and reference electrode that extend out from the device body that relays blood glucose fluctuations to the sensor in the device body and acts as an electrical reference for the current being measured by the glucose sensor, respectively. The implant is implanted intraperitoneally in the abdominal cavity. Prior to surgical implantation of the HD-XG implant, it must be switched to the ON mode with a magnet, implant operation must be verified audibly with a radio tuned to the low end of the AM band, and the glucose sensor must be hydrated by flooding the channel where the sensor lays with sterile saline approximately 15 min prior to implantation. The glucose sensor will be placed in the abdominal aorta and positioned so that the tip region of the sensor is between the renal arteries and the iliac bifurcation. The connector board will be attached to the back muscle. The implant portion of the device will be positioned inside the intraperitoneal cavity. The suture rib on the transmitter will be incorporated into the abdominal wall closure. New and sterile devices will be used for each surgery.

Glucose Telemeter HD-XG Implant Surgery: A DSI surgeon will use this approved protocol to perform aseptic surgery. DSI surgeons will be proficient in anesthesia and surgery. The surgeon will use sterile surgical techniques and use sterile gloves, mask, and gown. DSI surgeons will bring their own sterile instruments, which will be cleaned and sterilized between each animal by soaking in Benzalkonium Chloride for 15 min followed by a thorough rinse with sterile water. If necessary, glass bead sterilization will be used. The NHEERL staff will perform surgery preparation for the animals. These surgeons will have not been in contact with other rodents or another animal facility on the same day they enter the EPA animal facility. Surgeons will shower and change clothes and shoes before entering NHEERL facility.

We expect the surgeries to be performed as follows: The rats will be anesthetized using inhalation anesthesia using isoflurane and oxygen (induction – 2-4% isoflurane and 1-2 L/min of oxygen; maintenance – 1-3% isoflurane and 1-2L/min of oxygen). Animals will be checked for anesthetic level by lack of response to several firm toe pinches. Anesthetized rats will be injected with Buprenorphine analgesic subcutaneously at 0.02 mg/mL. Artificial tear ointment will be applied to prevent drying. Aseptic technique will be observed for the surgeries, including the use of sterilized instruments and suture material. Surgeons will wear mask, sterile surgical gloves, and may wear a sterile surgical gown. Surgeons will use autoclaved instruments. DSI surgeons will bring their own sterile instruments, which will be cleaned and sterilized between each animal by soaking in Benzalkonium Chloride for 15 min followed by a thorough rinse with sterile water.

Throughout the procedure including preparation, surgery, and post-operative care, supplemental warmth will be provided. During surgery, animals' temperature will be maintained by placement on recirculating

warm water blankets. Animals will have abdominal fur clipped, and will be scrubbed with surgical scrub and alcohol in alternating scrubs 3 times. The animal will be positioned in dorsal recumbency on the surgery table with the tail closest to the surgeon. The rat's limbs will be loosely taped to the table for support, and/or Press n seal will be used to keep the rat in place during surgery. The surgeon will direct staff to open sterile packs, sterile scalpel, and sterile drapes as needed. The rat will be draped in an aseptic fashion. The rat will be administered buprenorphine subcutaneously at 0.02 – 0.05 mg/kg and meloxicam subcutaneously at 2 mg/kg in a 2 ml saline pocket after induction of anesthesia.

Abdominal Aorta Cannulation: The stage of anesthesia will be re-checked. If there is no response to firm toe pinches, a 4-5 cm midline incision will be made through the skin of the abdomen. Blunt dissection will be used to gently separate the skin from the abdominal wall around the incision. Small surgical scissors or a scalpel blade will then be used to make a 4-5 cm midline incision through the abdominal wall with care taken to not damage the internal organs. Sterile cotton tipped applicators will be used to gently manipulate the intestines cranially and laterally to provide good visualization of the abdominal aorta where the left renal vein crosses over the aorta to the iliac bifurcation caudally. The intestines will be retracted using a sterile gauze sponge to allow access to the abdominal aorta. Once this gauze sponge retractor is in place, it will be thoroughly moistened with sterile saline. A sterile cotton tipped applicator will be used to carefully separate the overlying tissue from the surface of the aorta just caudal to the point where the left renal vein crosses over the aorta and just cranial to the iliac bifurcation. The sensor entry site will be just cranial to the iliac bifurcation. Using vessel dilators, the aorta will be carefully separated from the vena cava just caudal to the left renal vein. A piece of 4.0 or 5.0 suture will be placed between the vena cava and aorta so that the suture lies underneath the aorta. This suture will be used to temporarily occlude blood flow to allow introduction of the sensor into the vessel. Using the vessel dilators again, the aorta will be carefully separated from the vena cava just cranial to the iliac bifurcation. Another piece of 4.0 or 5.0 suture will be placed between the vena cava and aorta so that the suture lies underneath the aorta and will be used to temporarily occlude blood flow to allow introduction of the sensor into the vessel. Using cotton tipped applicators, the connective tissue and fat will be gently dissected from the lumbar muscle to the [surgeon's] left of the vena cava, either cranial or caudal to the ilio-lumbar vein. The space cleared should be large enough for the connector to be sutured to the lumbar muscle. The connector will sit to the left of the vena cava, as the surgeon looks at the animal [animal's right]. A sensor introducer will be prepared by bending a beveled tip of a 25-gauge syringe needle 90˚. Two to three gel-loading micropipette tips will be filled with Vetbond tissue adhesive and set aside. They will be used to dispense a very small amount of adhesive to seal the vessel. The implant will be removed from the sterile package and transferred to the sterile field. The tubing section of the sensor will be grasped with a pair of Vessel Cannulation Forceps with the working electrode of the sensor facing up. Gentle tension will be applied to both of the occlusion sutures using a hemostat to temporarily occlude blood flow in the aorta. The bent 25-gauge syringe needle will be used as the sensor introducer by piercing the artery 1-2 mm cranial to the iliac bifurcation. The sensor will be inserted upstream toward the heart. Once the sensor is inserted into the vessel, the needle will be withdrawn. The sensor will be advanced cranially so that the maximum amount of sensor is within the vessel. The aorta will be dried at the sensor entry site with cotton tip applicators and a very small amount of Vetbond tissue adhesive using the gel-loading micropipette tips will be applied. Once the Vetbond has visibly set, the tension on both the occlusion sutures will be slowly released to observe the sensor entry site for leakage. If leakage is observed, the vessel will be re-occluded, the site will be cleared of blood, and additional Vetbond will be applied to seal the leak. The caudal occlusion suture will be cut close to the aorta and carefully removed. The sensor will be anchored in place with two small fiber patches. One fiber patch will be placed on top of the sensor insertion site and secured to the sensor, vessel, and surrounding tissue using the Vetbond tissue adhesive on each of the 4 corners. The second fiber patch will be placed underneath the sensor insertion site facing in the opposite direction from the first. This patch will also be secured to the sensor, vessel, and surrounding tissue using the Vetbond tissue adhesive on each of the 4 corners. The cranial occlusion suture will be cut close to the aorta and carefully removed.

Connector Placement: A 4.0 non-absorbable suture will be placed through the back muscle near the ilio-lumbar vein with a small curved tapered needle leaving 3-4 cm long tails in the suture. A couple drops of sterile saline will be added to the back muscle before pulling the suture through to help prevent damage to the muscle. A loose square knot will be used to secure the suture to the back muscle. A second anchor suture will placed through the lumbar muscle below the first suture leaving one 3-4 cm tail and the needle

attached on the opposite side in a needle holder. All 4 tails of these anchor sutures will be spread out to the side so they will be easily accessible when the connector is in place. The tips of the vessel dilators will be placed on the suture rib on the underside of the connector, which will be gently flipped 180˚ cranially so that the suture rib is facing upwards away from the animal. The vessel cannulation forceps will be used to tuck the excess wire from the connector under the retraction gauze and intestines. The connector will be sutured in place to the lumbar muscles using square knots. A small drop of Vetbond will be applied to each of these knots.

Reference Electrode and Implant Placement: The gauze sponge retraction will be gently removed taking care to not dislodge the sensor or connector. The peritoneal cavity will be irrigated with warm, sterile saline and the intestines will be gently massaged back into place using cotton tipped applicators. The implant will be placed on top of the intestines parallel to the long axis of the body with the leads directed caudally and the suture rib directed ventrally. The reference lead will be placed into the abdominal cavity. Once the reference lead is in place, the signal can be verified. A simple interrupted suture using non-absorbable material will be placed at the cranial aspect of the body wall incision, incorporating the most cranial rib of the implant body in this suture. A suture will be placed through the underside of the abdominal wall about 2 cm from the abdominal incision and a square knot will be tied leaving 3-4 cm of suture tail on one end and the suture with the needle still attached on the other end. The suture sleeve on the reference electrode will be aligned with this knot. The suture tails will be tied around the suture sleeve securing the reference electrode to the abdominal wall and the suture tails will be cut short. Another suture will be placed through the underside of the abdominal wall that aligns with the second notch in the suture sleeve and secured with a square knot. These suture tails will be tied around the suture sleeve with the tails cut short.

Abdominal Closure: The abdomen will be closed using 4.0 or 5.0 non-absorbable suture with a simple interrupted pattern incorporating the remaining suture ribs on the implant into the closure. The skin incision will be closed using wound clips or 4.0 or 5.0 absorbable or non-absorbable suture. GLUture will be applied to the skin over the wound clips or suture. Bupivacaine local anesthesia (1 mg/kg) will be administered subcutaneously.

Surgical Recovery: Surgical anesthesia will be discontinued. Supplemental warmth will be maintained throughout anesthetic recovery. Post-surgical analgesia will be administered. Saline (15 mL/kg) will be given subcutaneously for fluid support and will be repeated as necessary twice daily for 3 days. Animals will be monitored closely for return of normal postures and behaviors.

b. Anesthetic regimen (Drugs, dosages, volume, route of administration and delivery schedule). The use of paralytic or neuromuscular blocking agents w/o anesthesia is prohibited:

The rats will be anesthetized using inhalation anesthesia using isoflurane and oxygen. Induction of the anesthetic state will be done using 2-4% isoflurane and 1-2 L/min of oxygen. Maintenance of the anesthetic state will be maintained using 1-3% isoflurane and 1-2 L/min of oxygen delivered via nose cone. Animals will be checked for anesthetic level by lack of response to several firm toe pinches.

c. Postoperative care (thermal support, special feeding, responsible personnel, removal of sutures/staples, frequency and duration of monitoring including weekend and holiday care):

After surgery, rats will be continuously monitored until fully awake and placed on a heating pad and/or other heat source combination such as forced air warmer, space gels, bubble wrap, and infrared heating pad to maintain body temperature. Powdered food provided in feeding cups and regular feed offered in bowls on the floor and the food hopper will be provided post-surgery for all animals for at least 7-10 days post-surgery. After complete awakening, animals will be injected with Meloxicam (1 mg/kg, subcutaneously) for analgesia and then transferred to their home cages. Meloxicam will be given once daily at 24h in between for 3 full days after surgery. Buprenorphine will be given for analgesia for at least 2 full days given 6-8h after surgery then every 8-12h for the next 2 days. The dose is 0.02-0.05 mg/kg given subcutaneously. Although no deaths are anticipated from this procedure, rats will be closely monitored during the postoperative period. After the surgery, animals will be allowed to recover from surgery for 6 days and then used for the study. No other special husbandry requirements are needed for implanted rats. We will closely monitor these rats and will provide additional analgesic treatment as recommended by attending veterinarian.

Exemption 6Exemption 6Exemption 6Exemption 6 will be monitoring animals twice

daily after their surgery (during recovery and experimentation periods including weekends). It is not expected that animals will suffer dehydration or weight loss issues from this type of surgery. Nevertheless, the attending Veterinarian will be notified immediately and if advised by the attending veterinarian, subcutaneous saline bolus will be administered as suggested (10-30 ml/kg).

We do not anticipate incidences of dehiscence on the abdominal surface of the animals. Nevertheless, the attending veterinarian will be notified if any incidences of dehiscence are observed, and the recommendations will be followed for the follow-up procedures and treatments. Dehiscence might require flushing of an open incision with sterile saline followed by a closure of wound using sterile surgical suture or application of surgical glue. This procedure will be performed in Exemption 6 Exemption 6 Exemption 6 procedure room using aseptic technique. Based on the recommendation of attending veterinarian, we will consider injectable analgesic and/or antibiotic treatment.

Attending veterinarian will be consulted and recommendations will be followed for animals displaying unexpected weight loss of 10% or greater. All animals will be double-housed with a non-telemetered cagemate with Alpha-dri bedding in A building during non-exposure periods.

d. Post operative analgesics (drugs, dosage, and volume and route of administration, frequency):
Upon awakening, rats will be injected subcutaneously with Meloxicam analgesic, 1 mg/kg dose in pharmaceutical grade saline. Meloxicam will be given once daily at 24h in between for 3 full days after surgery. Buprenorphine will be given for analgesia for at least 2 full days given 6-8h after the first dose then every 8-12h for the next 2 days. The dose is 0.02-0.05 mg/kg given subcutaneously. An additional dose of Buprenorphine will be given if advised by attending veterinarian. If infection or swelling or redness is observed in rats, we will consult attending veterinarian to suggest appropriate protocol for injectable antibiotic treatment.

e. Will any animal be subject to more than one surgical procedure over the course of its lifetime, either here at NHEERL or elsewhere?
○ Yes ● No

f. Identify any surgical procedures performed at other institutions or by vendors:
Implant surgeries will be performed by the DSI surgeons at the EPA facility, and details are provided in B.7.a.-d.

8. Humane interventions (for treatments/procedures in all categories).

a. What resultant effects, if any, do the investigators expect to see following procedures or treatment? Please include transitory as well as permanent effects. Examples might include lethargy, ataxia, salivation or tremors. Indicate the expected duration of these effects.
No deleterious effects of these implant surgeries are expected. Unexpected weight loss of 10% or greater or visual signs of distress will result in immediate notification to the attending Veterinarian and consideration for euthanasia. In case any complications are observed, we will consult with attending veterinarian and follow recommended protocol. These animals will be exposed to ozone at concentrations previously used in our laboratory and no ozone exposure-related complications are expected.

b. State the criteria for determining temporary or permanent removal of animals from the study. Describe actions to be taken in the event of deleterious effects from procedures or chemical exposures. Describe actions to be taken in the event of clinical health problems not caused by procedures or exposures.
If weight loss of >10% occurs overnight, animals will be isolated in a clean, control atmosphere and observed for recovery trend. Any animals displaying signs of illness (huddling, isolation with ruffled exterior, shivering, development of hindered movement, etc.) will be considered for permanent removal as per advice of the staff veterinarian. Visual inspection of labored breathing and isolation will be carefully monitored and if noted, the advice of the attending veterinarian will be followed for further action and possible euthanasia.

9. Alternatives to pain and distress (Category D and E Procedures only). Provide narrative regarding the sources consulted to ascertain whether acceptable alternatives exist for potentially painful/distressful procedures. Include databases searched or other sources consulted, the date of the search and years covered by the search, and key words and/or search strategy used. Assistance with searches is available through the EPA Library Staff.

We have read many papers published in the past 20 years and have searched Pubmed and Google Scholar (keywords: air pollution, glucose monitors, and rat, searched in March 2017) to look for alternatives to implant glucose monitors to study pollution health effects. Static glucose measurements post-exposure have been conducted in several studies using blood collected from a single tail prick. Although this is an acceptable, safe way to measure blood glucose levels post-exposure, it does not provide the continuous, dynamic blood glucose levels prior to, during, and following pollution exposure. Implanting these glucose monitors is the only means available for these types of measurements. Therefore, it is necessary to use these HD-XG glucose telemeters for understanding the mechanisms by which pollutants alter glucose homeostasis.

SECTION C - Animal requirements

Describe the following animal requirements :

1. Indicate the number of animals required over the study period for this protocol. Please enter numbers only.

a. Animals to be purchased from a Vendor for this study: 84

b. Animals to be transferred from another LAPR:

LAPR Number that is the source of this transfer:

c. Animals to be transferred from another source:

d. Offspring produced onsite (used for data collection and/or weaned):

e. TOTAL NUMBER of animals for duration of the LAPR 84

2. Species (limited to one per LAPR): Rat(s)

3. Strain: WKY rat(s)

Describe special requirements for animals with altered physiological responses (e.g., genetically altered, aged)

N/A

4. Sources of animals:

Charles River Laboratories, Inc.

5. Provide room numbers where various procedures will be performed on animals:

1. Exemption 6, or other available room on 5th floor - housing of rats
2. Exemption 6 - whole-body plethysmography, GTT, PTT, ITT
3. Third flood procedure room Exemption 6 - surgery
4. Exemption 6 - whole body ozone exposures
5. Exemption 6 - necropsy

6. Will any animals be housed in areas other than the animal facility longer than 12 hours? If so, state location. Such areas require prior IACUC approval as a satellite facility before LAPR can be reviewed.

No *Room Numbers:*

7. Describe any transportation and containment methods involved in moving animals between EPA buildings, or between EPA and other institutions (excluding any commercial shipments)

n/a

8. Describe any unusual housing or husbandry requirements, or acclimation requirements. Justify any treatment beginning less than 3 days after arrival.

none

9. Describe special assistance requested of the animal contract staff, including procedures and dosing. NOTE, this request must be submitted separately to the Animal Resources Program Office (ARPO)

The days of surgical procedures, we will submit a request for technical assistance during animal recovery from anesthesia. There will be a requirement for bottled water for animals. Additional assistance will be requested as needed.

10. Housing and Enrichment.

The IACUC encourages the use of environmental enrichment whenever possible (see IACUC website for details). Provide details on how the animals will be housed, including type of cage (e.g., solid bottom or wire screen), bedding material, number of animals per cage, and environmental enrichment. Note that housing rodents individually without environmental enrichment requires justification.

All implanted animals in Experiment #1 (n=8) will be double housed with a non-telemetered cagemate in solid bottom caging with Alpha-dri chips bedding in A building during non-exposure periods for real-time, live recording of blood glucose data through receivers placed under each cage. All animals in Experiment #2 will be double housed. Environmental enrichment including crinkled paper (EnviroDry) will be provided to allow the rats to nest. If for some reason (i.e. behavioral/fighting or clinical), rats will be singly housed and will be provided with appropriate environmental enrichment.

SECTION D - Agents Administered to Animals

1. Identify all hazardous and non-hazardous agents to be administered to living animals. For agents requiring a Health and Safety Research Protocol (HSRP), provide the title of the approved HSRP for each such agent. If no protocol is required for an agent deemed potentially hazardous (e.g. nanoparticles, recombinant DNA), describe the safety precautions to be used.
Provide maximum dosing levels and route-appropriate LD50s (where available) for each agent used for dosing.

1) Isoflurane: The rats will be anesthetized using isoflurane and oxygen inhalation anesthesia. Induction of the anesthetic state will be done using 2-4% isoflurane and 1-2 L/min of oxygen. Maintenance of the anesthetic state will be maintained using 1-3% isoflurane and 1-2L/min of oxygen delivered via nose cone. Animals will be checked for anesthetic level by lack of response to several firm toe pinches.

2) Ozone inhalation exposures: Ozone exposure will occur in whole-body exposure chambers to a maximum of 0.8 ppm concentration. The LC50 for ozone is 4.8 ppm in rats (4800 ppb/4h/inhalation/rat). HSRP copy attached (#778) (Title: Small Animal Inhalation Exposures to Nitrogen Dioxide and Ozone).

3) Buprenorphine: This is a veterinary grade analgesic widely used in experimental studies during surgery. Rats will receive a total of three or more subcutaneous injections of Buprenorphine after the surgery at a recommended dose level of 0.02 mg/kg/mL in saline. Mouse oral LD50 for buprenorphine is 800 mg/kg.

4) Meloxicam: This analgesic will be subcutaneously injected once in rats after they are awake from anesthesia. The recommended dose will be 0.2 mg/kg/mL in saline. This is a veterinary grade analgesic widely used in experimental studies during surgery. Rat oral LD50 for meloxicam is 83.5 mg/kg.

5) Glucose: During GTT, pharmaceutical grade glucose will be injected intraperitoneally (maximum of 2 g/kg body weight/10 mL). The 10 mL/kg body weight volume has been used in published studies for rats.

6) Sodium Pyruvate (not available in pharmaceutical grade): 1 g sodium pyruvate/kg body weight/2 mL pharmaceutical grade saline will be injected intraperitoneally in rats. LD50 information is not available.

7) HumulinR Insulin: Pharmacological grade recombinant human insulin. Injectable preparation will be diluted with pharmacological grade saline to derive 0.75 U/mL and injected in rats intraperitoneally during ITT. Rat LD50 is reported as 10 U/kg subcutaneously in the MSDS.

Researchers will handle all agents in accordance with good industrial hygiene and safety practices. Lab coat, safety glasses, and gloves will be worn when handling these chemicals. Drug preparations will be done in chemical safety hood.

2. Describe compounds to be administered to animals.

a. Are all substances pharmaceutical grade? If not, provide a scientific justification for the use of non pharmaceutical grade compounds.

Among the substances injected in animals, only sodium pyruvate is not pharmaceutical grade but is the highest purity chemical prepared in pharmaceutical grade saline. We have used this preparation in previously published studies. The use of these research grade drugs is necessary for comparing research results from our studies to those that have been published using these drugs.

b. Describe any plans to administer human or animal tissues, blood or body fluids to the animals in the LAPR. Provide information to assure that such material is pathogen free. Indicate what safety precautions are necessary for handling the material.

none

c. Provide a statement regarding any safety precautions necessary for handling any of these materials.

Researchers will handle all agents in accordance with good industrial hygiene and safety practices. Lab coat, safety glasses, and gloves will be worn when handling these chemicals. Drug preparations will be done in
chemical safety hood.

NOTE: Any unresolved health/safety questions which arise during IACUC review of this LAPR will require consultation with the Safety, Health, and Environmental Management Office.

SECTION E - Personnel Training and Experience

1. Identify all project personnel conducting animal experimentation. Specify the techniques for which they have responsibility, and their relevant training and experience. Additional personnel may be added to the table below as a group (by Division) for Category C procedures. By so doing you are giving assurance that these personnel have received all required training and are qualified to perform the Category C techniques requested.

Use this area to type in additional personnel information not available in the table drop-down lists:

Hint: *The names in the first 2 lines of the table below are filled automatically from the Principal Investigator & Alternate Contact fields. A new line will be made available when a name is selected & upon leaving the name field (i.e. tabbing or clicking in another field).*

NAME	ROLE	SPECIFIC RESPONSIBILITY	RELEVANT TRAINING
Exemption 6	Principal Investigator	Plan study, prepare protocols, and oversee the experiment. Assist in animal handling, testing, and necropsy.	Twenty five years of experience working with rats at EPA and other institutions; all relevant NHEERL required training completed.

Exemption 6	Associate Principal Investigator	Plan study, prepare protocols, and oversee the experiment. Assist in animal handling, testing, and necropsy.	Four years of experience working with rats at EPA and other institutions; all relevant NHEERL required training completed.
Exemption 6 Exemption 6 Exemption 6 Exemption 6	Technical Staff	Plan study, prepare protocols, and oversee the experiment. Assist in animal handling, testing, and necropsy.	Twenty years of experience working with rats at EPA and other institutions; all relevant NHEERL required training completed.
Exemption 6	Student	Assist in animal handling, testing, and necropsy	All relevant NHEERL required training completed. He will be mentored and supervised by the principal investigator during animal handling.
Exemption 6	Technical Staff	Assist in animal handling during in-life testing, exposure and plethysmography	Twenty years of experience working with rats at EPA and other institutions; all relevant NHEERL required training completed.
Exemption 6	Post-Doc	Assist in animal handling, testing, and necropsy	Five years of experience working with rats at EPA and other institutions; all relevant NHEERL required training completed.
Exemption 6	Associate Principal Investigator	Assist in glucose monitoring	Ten years of experience working with rats at EPA and other institutions; all relevant NHEERL required training completed.
Exemption 6	Associate Principal Investigator	Assist in glucose monitoring	Ten years of experience working with rats at EPA and other institutions; all relevant NHEERL required training completed.
Exemption 6	Associate Principal Investigator	Assist in animal handling during in-life testing, exposure and plethysmography	Twenty years of experience working with rats at EPA and other institutions; all relevant NHEERL required training completed.
Exemption 6	Post-Doc	Assist in animal handling during in-life testing, exposure and plethysmography	Five years of experience working with rats at EPA and other institutions; all relevant NHEERL required training completed.
RTP-NHEERL	Tech Support	Category C Procedures	All NHEERL required training is complete.

SECTION F - Animal Breeding Colonies

This section pertains to the breeding of animals for maintenance of ongoing animal colonies. Do not include breeding that is part of experimentation and accountable under Section C.

Describe:

1. Estimated number of breeding pairs and liveborn per year	None
2. Breeding protocols and recordkeeping	n/a
3. Methods for monitoring genetic stability	n/a
4. Disposition of all offspring and retired breeders that are not used in accordance with the procedures described in this LAPR	n/a

SECTION G - Euthanasia

1. When will the animals be euthanized relative to experimental procedures?

Animals will necropsied for blood sample and tissue collections following terminal euthanasia, at designated times after air or ozone exposure and within 70 days of surgical intervention.

2. Describe the euthanasia techniques:

Method(s): Euthanasia plus exsanguination
Agent(s): Pentobarbital injectable preparation, diluted with sterile saline to achieve maximum of 200 mg/mL concentration.
Dose (mg/kg): 200-250 mg pentobarbital/kg.
Volume: 1.0 – 3.0 mL/kg as needed
Route: Intraperitoneal

Source(s) of information used to select the above agents/methods:

Veterinary Staff
IACUC

3. Provide justification and references for any euthanasia agent or method that is not consistent with recommendations of the American Veterinary Medical Association (AVMA) Guidelines for Euthanasia (e.g., cervical dislocation or decapitation without anesthesia; cervical dislocation in rodents weighing more than 200 grams).

None

4. Describe how death is to be confirmed.

Vital organ section

SECTION H - Disposition of Used and Unused Animals

Describe the disposition of any animals remaining after project completion.

Transferred to another study

The IACUC encourages investigators to reduce the overall number of animals used at NHEERL. Would you consider transferring any unused animals from this LAPR to another approved LAPR?

● Yes ○ No

SECTION I - Assurances

1. Animals will not be used in any manner beyond that described in this application without first obtaining formal approval of the IACUC.
2. All individuals involved in this project have access to this application, are aware of all EPA policies on animal care and use, and are appropriately trained and qualified to perform the techniques described.
3. Thorough consideration of the three "R"'s (Replacement, Reduction, Refinement) has been given, as applicable, to a. the use of animals, and b. procedures causing pain or distress (with or without analgesia/anesthesia), including death as an endpoint. The minimum number of animals required to obtain valid experimental results will be used.
4. The Attending Veterinarian has been consulted in regard to any planned experimentation involving pain or distress to animals.
5. The IACUC and Attending Veterinarian will be promptly notified of any unexpected study results that impact the animals' well-being, including morbidity, mortality and any occurrences of clinical symptoms which may cause pain or indicate distress.
6. All procedures involving hazardous agents will be conducted in accordance with practices approved by the Safety, Health, and Environmental Management Office.

7. I certify that I am familiar with and will comply with all pertinent institutional, state and federal rules and policies.
8. The IACUC has oversight responsibilities for animal care and use, and may request consultation or feedback regarding the conduct of in vivo procedures, progress and accomplishments, and any problems encountered.

EPA Principal Investigator	Certification Signature Date
Exemption 6 Exemption 6	03/29/2017

Submitted: 03/29/2017

Certification:

Certification by EPA Supervisor (Branch Chief or Division Director) that the project described herein has been reviewed and approved on the basis of scientific merit:

Branch Chief/Division Director	***Approval Date***	***Phone Number***	***Division***	***Mail Drop***
Exemption 6	03/29/2017 by Exemption 6 Exemption 6 RTP/USEPA/US	Exemption 6 ***Lotus Notes Address*** Exemption 6 Exemption 6 /RTP/USEPA/US	EPHD ***Branch*** CIB	MD ***Submitted to Branch Chief for Approval*** 03/29/2017 08:31 AM

ATTACHMENTS

Experimental Design for LAPR - Exemption 6 Exemption 6 .docx 20-04-001 PI resp 2 prescreen.pdf 20-04-001 prescreen 2.pdf

20-04-001 prescreen 1.pdf HSRP form NO2 and ozone exposures 131112 final._778.pdf

Surgical Manual for Glucose Telemeter Implants.pdf

Actions

First Update notification sent: 03/02/2018
Second Update notification sent:
First 2nd Annual notification sent:

Second 2nd Annual notification sent:

1st Expiration notification sent:
2nd Expiration notification sent:

History Log: